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DIALOG 6479716, embase no. 87217030; M.  
ITO et al.: "Synergistic antiproliferative ef-  
fect of Interferon-beta in combination with  
bleomycin or neocarcinostatin on HeLa cells  
in culture: additive effect when combined  
with adriamycin or mitomycin C", & J. INTER-  
FERON RES. (USA), 1987, 7/4 (419-425)

㉙ Proprietor: CETUS CORPORATION  
1400 Fifty-Third Street  
Emeryville California 94608(US)

㉚ Inventor: Zimmermann, Robert  
3912 N. Peardale Drive  
Lafayette California 94549(US)  
Inventor: Marafino, Benedict Joseph, Jr.  
10 Park Ridge Drive No. 10  
San Francisco California 94131(US)

㉛ Representative: Vossius & Partner  
Siebertstrasse 4 P.O. Box 86 07 67  
W-8000 München 86(DE)

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**Description**

This invention relates to a composition suitable for administration to mammalian hosts as a therapeutic formulation. More particularly, this invention relates to a combination therapy for free-radical bodily damage employing a lymphokine or cytotoxin such as tumor necrosis factor (TNF) and a biological modifier consisting of either one or more free radical scavengers that protect against damage caused by free-radical generation, or that selectively increase the susceptibility of a tumor to radical damage by depleting or reducing its radical scavenging capacity, or an inhibitor of one or both of the cyclooxygenase or lipoxygenase pathways of arachidonic acid metabolism.

5 Lymphokines and cytotoxins, such as interleukin-2, interferon-alpha, interferon-gamma, colony stimulating factor, and tumor necrosis factor, are proteins secreted by T cells and/or macrophages upon activation by antigens or lectins. Interleukin-2 (IL-2), a lymphokine which is produced by normal peripheral blood lymphocytes and induces proliferation of antigen or mitogen stimulated T cells after exposure to plant lectins, antigens, or other stimuli, was first described by Morgan, D.A. et al., Science (1976) 193:1007-1008.

10 Then called T cell growth factor because of its ability to induce proliferation of stimulated T lymphocytes, it is now recognized that in addition to its growth factor properties it modulates a variety of functions of immune system cells in vitro and in vivo and has been renamed interleukin-2 (IL-2). IL-2 is one of several lymphocyte-produced, messenger-regulatory molecules which mediate immunocyte interactions and functions.

15 20 Tumor necrosis factor (TNF) was first described by Carswell et al., Proc. Natl. Acad. Sci. USA (1975) 72:3666-3670 as an endotoxin-induced serum factor which causes necrosis of chemically transformed tumor cells when growing in mice. Human TNF is known to be cytotoxic to neoplastic cells, and has been produced in recombinant form. See Pennica et al., Nature (London) (1984) 312:724-729 and Shirai et al., Nature (London) (1985) 313:803-806, Wang et al., Science (1985) 228:149-154.

25 25 Interferons (IFN) constitute a group of naturally occurring proteins which are known to exhibit anti-viral, anti-tumor and immunoregulatory behavior. Two types of IFN have been identified based on differences in their observed biological properties and molecular structures: Type I and Type II. Beta-interferon (IFN- $\beta$ ) is a Type I IFN which can be induced in fibroblasts by viral challenge and contains about 165 amino acids. IFN- $\alpha$  is also a Type I IFN inducible in leukocytes, and IFN- $\gamma$  is a Type II IFN which is induced in lymphocytes in response to specific mitogenic stimuli and contains 146 amino acids.

30 35 lymphocytes in response to specific mitogenic stimuli and contains 146 amino acids. Combination chemotherapy using two or more anti-cancer drugs to treat malignant tumors in humans is currently in use in research and in the clinic. The anti-cancer drugs may be antimetabolites, alkylating agents, antibiotics, general poisons, etc. Combinations of drugs are administered in an attempt to obtain a synergistic cytotoxic effect on most cancers, e.g., carcinomas, melanomas, lymphomas and sarcomas, and to reduce or eliminate emergence of drug-resistant cells and to reduce side effects to each drug.

40 45 For example, it is known that IL-2 may be used with IFN- $\gamma$  to treat tumor-bearing hosts with synergistic results [EP-A-0 149 551, published July 24, 1985 (Genentech) and DE-A-3411184, published October 31, 1985 (Deut. Rotes Kreuz)] or with augmentation of natural killer activity [Svedersky et al., J. Immunol. - 1984, 133:714-718 and Shalaby et al., J. Interferon Res. (1985), 5:571-581]. In addition, U.S. Statutory Invention Reg. No. H22, published February 4, 1986 to Creasey et al., discloses a composition exhibiting a synergistic cytotoxic effect in combination therapy of certain breast cancer and myeloma cell lines using synergistically effective amounts of 5-fluorouracil and human recombinant beta-interferon. Furthermore, enhanced anti-tumor activity has been observed using IFN- $\gamma$  in combination with TNF and chemotherapeutic agents, Svedersky et al., Internl. J. of Immunopharmac. (1985) 7:330.

50 55 An understanding of the mechanisms of action of various lymphokines and cytotoxins and the basis of tumor cell sensitivity to such proteins would facilitate the clinical investigation and the design of clinical trials of these therapeutic agents. For example, TNF, which is produced primarily by macrophages, has shown an apparent selectivity for many tumor cells, but not normal cells, in its cytotoxic or cytostatic activities. See, e.g., Carswell et al., supra, Wang et al., supra, Ruff and Gifford in Lymphokines, Volume 2, ed. Pick, E. (Academic Press, Inc., NY, NY, 1981), pp. 235-272, Beutler and Cerami, Nature (1986) 320:584-588, and Urban et al., Proc. Natl. Acad. Sci. USA (1986) 83:5233-5237, and the references cited therein. The basis for this selective killing of tumor cells is known not to be due to receptor absence, inasmuch as TNF cells, such as human diploid fibroblasts, have sufficient numbers of high affinity receptors, internalize TNF, and degrade it in an apparently similar fashion as TNF cells do. Tsujimoto, M. et al., Proc. Natl. Acad. Sci. USA (1985) 82:7626-7630.

55 Interleukin-1 alone has a protective effect in a model of free radical dependent tissue injury, Neta et al., J. Immunol. (1986) 136:2483-2485. In addition, it has been found that TNF- $\alpha$  and IFN- $\gamma$  induce neutrophils from normal and chronic granulomatous-disease patients to release superoxide, Palladino et al., Clin. Res. -

(1986) 34:502 and Palladino et al., Ped. Res. (1986) 20:302.

The biological activity of both oxygen-free radical species and related polyunsaturated fatty acid lipid peroxidation products has been well established. For example, the generation of reactive radical species has been found to be involved in the cytotoxic effects of ionizing radiation [see, e.g., Petkau, Acta Physiol.

- Scand. Suppl. (1980) 492:81-90 and Biaglow et al., Radiat. Res. (1983) 95:437-455], various chemotherapeutic agents [see, e.g., Tomasz, Chem. Biol. Interact. (1976) 13:89-97, Lown and Sim, Biochem. Biophys. Res. Commun. (1977) 77:1150-1157 and Borek and Troll, Proc. Natl. Acad. Sci. USA (1983) 80:1304-1307], and a variety of other biological processes, including aging, and the initiation and promotion stages of experimental carcinogenesis [see, e.g., DiGuiseppe and Fridovich, CRC Crit. Rev. Toxicol. (1984) 12:315-342 and Slater, Biochem. J. (1984) 222:1-15]. The generation and release of reactive free radicals in the respiratory burst phenomenon used by various cells of the immune system is a well known mechanism of foreign target destruction. See, e.g., Bus and Gibson in Rev. Biochem. Toxicol., eds. Hodgson et al. (Elsevier, North Holland, 1979), pp. 125-149 and Badwey and Karnovsky, Ann. Rev. Biochem. (1980) 49:695-726.

In aerobes, a variety of radical scavenging mechanisms have evolved at both the cellular and organismal level that confer protection from potentially lethal reactive oxygen species, such as the hydroxy radical, superoxide anion, and hydrogen peroxide. See, e.g., DiGuiseppe and Fridovich, supra, Slater, supra, and Bus and Gibson, supra. Importantly, oxygen radicals can initiate longer-lived chain reactions of lipid peroxidation that can be propagated from cell to cell. These peroxidation products are capable of damaging cellular DNA, RNA, protein, and cellular phospholipids. See, e.g., Slater, supra, Bus, supra, Moody and Hassan, Proc. Natl. Acad. Sci. USA (1982) 79:2855-2859, Lesko et al., Biochemistry (1980) 19:3023-3028, and Cerutti et al. in Genes and Proteins in Oncogenesis (Academic Press, NY, 1983), pp. 55-67. The protective cellular mechanisms against this kind of damage include anti-oxidants and radical scavengers in both the lipid (e.g.,  $\alpha$ -tocopherol,  $\beta$ -carotene) and aqueous (e.g., glutathione and ascorbic acid) phases of cells, as well as enzymes such as superoxide dismutase and catalase. See, e.g., Fridovich, Science (1978) 201:875-880 and Meister and Anderson, Ann. Rev. Biochem. (1983) 52:711-760. The high plasma uric acid level found in humans has also been shown to be a major radical protective factor. Ames et al., Proc. Natl. Acad. Sci. USA (1981) 78:6858-6862.

Glutathione (GSH) and related cellular sulfhydryl compounds represent one of the major mechanisms of detoxification of electrophilic metabolites of xenobiotics and oxygen/lipid radical species, Meister and Anderson, supra. Inhibition of free radicals is postulated as the way in which certain radioprotectors, such as the free radical scavengers cysteine and GSH, operate. GSH becomes oxidized to contain a dithio group as well as to protein-mixed disulfides, when cells are exposed to oxygen-generating compounds or other oxidative stresses. See Adams et al., J. Pharmacol. Exp. Ther. (1983) 227:749-754. Therefore, the content of oxidized GSH is one important indicator of either the type of damage to which a cell has been exposed or of its ability to protect itself from oxidative damage. Buthionine sulphoximine has been shown to be an inhibitor of GSH biosynthesis. See Minchinton et al., Int. J. Radiation Oncology Biol. Phys. (1984) 10:1261-1264.

A protein called monocyte cell line cytotoxin (MCCT) was characterized and the inhibitory effects of various protease inhibitors and hydrogen peroxide scavengers on MCCT activity were studied, Armstrong et al., J.N.C.I. (1985) 74:1-9. In addition, it was found that various hydroxyl radical scavengers inhibited production of a lymphotoxin. See Kobayashi et al., J. Biochem. (Tokyo) (1984) 95:1775-1782. Finally, methisoprinol, a purine derivative, has been shown to increase the production of lymphotoxin, which is a lymphokine, Morin and Ballet, Allergol. Immunopathol. (1982) 10:109-114.

Marcus et al., Cancer Research, 47:4208-4212 (1987) discloses use of vitamin C and IL-2.

Arrick et al., J. Clin. Invest. 71:258-267 (1983) discloses that inhibition of glutathione synthesis [e.g., by buthionine sulfoximine (BSO)] enhances lysis of tumor cells by antineoplastic agents.

Romine and Kessel, Biochem. Pharmacol. (UK) (1986) 35:3323-3326 discloses the role of intracellular glutathione as a determinant of responsiveness to antitumor drugs.

Ono et al., Br. J. Cancer (UK) (1986) 54:749-754 discloses the combined effect of BSO and cyclophosphamide on murine tumors and bone marrow.

Hamilton et al., Biochem. Pharmacol. (July 15, 1985) 34:2583-2586 discloses the enhancement of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and drug-sensitive carcinoma cell lines by use of BSO.

Andrews et al., Cancer Res. (Dec. 1985) 45:6250-6253 discloses the differential potentiation of alkylating and platinating agent cytotoxicity in human ovarian carcinoma cells by glutathione depletion.

Russo et al., Cancer Res. (June 1986) 46:2845-2848 discloses selective modulation of glutathione levels in human normal versus tumor cells and differential response to chemotherapy drugs.

Tew et al., Cancer Treatment Rep. (June 1986) 70:715-720 discloses the relationship of glutathione depletion to the antimitotic properties of estramustine.

Russo et al., Int. J. Radiat. Oncol. Biol. Phys. (Aug. 1986) 12:1347-1354 discloses the roles of intracellular glutathione in antineoplastic chemotherapy.

Dorr et al., Invest. New Drugs (1986) 4:305-313 discloses the cytotoxic effects of glutathione synthesis inhibition by BSO on human and murine tumor cells.

Green et al. Cancer Res. (Nov. 1984) 44:5427-5431 discloses that incubation of cells in the presence of BSO resulted in markedly increased (synergistic) melphalan cytotoxicity, and Oxols, Semin. Oncol. (Sept. 1985) 12:7-11 discloses that BSO increases the cytotoxicity of melphalan and cisplatin.

Oxols et al., Dev. Oncol. (1986) 47:277-293 discloses the effect of BSO on the efficacy of antitumor drugs.

Crook et al., Cancer Res. (1986) 46:5035-5038 discloses that BSO enhances the cytotoxicity of cyclophosphamide. Hodgkiss et al., Biochem. Pharmacol. (1985) 34:2175-2178 discloses use of BSO to enhance the cytotoxicity of nitroaromatic compounds. Somfai-Relle et al., Biochem. Pharmacol. (1984) 33:485-490 discloses that BSO sensitizes murine tumor cells to L-phenylalanine mustard.

EP-A-0 177 342 discloses pharmaceutically acceptable formulations designed for the purpose of administering certain amphophilic proteins combined with lipophilic vehicles and GI tract adsorption enhancing agents in oral dosage form. Said formulations do not comprise biological modifiers as free radical scavengers or metabolic inhibitors.

Accordingly, the present invention is based on the finding that the therapeutic index of a lymphokine or cytotoxin can be enhanced in *in vitro* and in *in vivo* systems by concomitantly or separately treating the host with a free radical scavenger such as a radiation protector or a metabolic inhibitor in amounts that increase the efficacy and/or decrease the toxicity of the lymphokine or cytotoxin.

More specifically, the present invention is directed to a composition suitable for administration to mammalian hosts for therapeutic or prophylactic treatment of biological damage to mammalian hosts caused by free radical production which composition comprises a mixture, in pharmacologically effective amounts, of at least one lymphokine or cytotoxin from a mammalian species and at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor.

Preferably, the lymphokine or cytotoxin is tumor necrosis factor or interleukin-2 and the free radical scavenger or metabolic inhibitor is uric acid, buthionine sulphoximine, vitamin C, indomethacin, ibuprofen, N-acetyl cysteine, or aspirin.

Without intent to be limited to any one theory, it is believed that the sensitivity of damaged cells, such as tumorous, infected, or irradiated cells, to a lymphokine or cytotoxin such as TNF is dependent on free-radical scavenging capacity. It is also believed that activation of the arachidonic acid cascade may be involved in the mechanism of action of the lymphokine or cytotoxin, which can produce lipid peroxidation and other associated radical species, as well as the biologically active metabolites of the lipoxygenase and cyclooxygenase pathways.

As used herein, the term "lymphokine" refers to low molecular weight proteins that are secreted by T cells and/or macrophages when antigens or lectins stimulate T cell or macrophage growth or activation. The term "cytotoxin" refers to any protein that activates effector cells that kill foreign agents such as pathogens in the cell. Examples of such lymphokines and cytotoxins include, but are not limited to, interferons [e.g., interferon-alpha, (IFN- $\alpha$ ), interferon-beta, (IFN- $\beta$ ), and interferon-gamma, (IFN- $\gamma$ )], interleukins [e.g., interleukin-1 (IL-1), Interleukin-2 (IL-2), interleukin-3 (IL-3), and interleukin-4 (IL-4)], tumor necrosis factor-interleukin-1 (IL-1), Interleukin-2 (IL-2), interleukin-3 (IL-3), and interleukin-4 (IL-4)], tumor necrosis factor-beta (TNF- $\beta$ ) (also called lymphotoxin), a colony stimulating factor (e.g. alpha (TNF- $\alpha$ ), tumor necrosis factor-beta (TNF- $\beta$ )) (also called lymphotoxin), a colony stimulating factor (e.g. CSF-1, CSF-G or CSF-GM), chemotaxins, migration inhibitory activity factor (MIF), macrophage-activating factor (MAF), NK cell activating factor, T cell replacing factor, leukocyte-inhibitory factor (LIF), other lymphotaxins, osteoclast-activating factor (OAF), soluble immune response suppressor (SIRS), growth-stimulating factor and a monocyte growth factor. Preferably, the lymphokine or cytotoxin is an interleukin (more preferably IL-2), an interferon (more preferably IFN- $\beta$ ), TNF- $\alpha$  or - $\beta$ , or a colony stimulating factor (more preferably CSF-1). The most preferred herein is TNF- $\alpha$ .

As used herein, the term "recombinant" refers to lymphokines or cytotoxins produced by recombinant DNA techniques wherein generally the gene coding for the lymphokine or cytotoxin is cloned by known DNA techniques and inserted into a template, the gene showing complementarity to the human lymphokine or cytotoxin cDNA is inserted into a suitable DNA vector such as a bacterial plasmid, preferably an *E. coli* plasmid, to obtain a recombinant plasmid, and the plasmid is used to transform a suitable host. The gene is expressed in the host to produce the recombinant protein. Examples of suitable recombinant plasmids for this purpose include pBR322, pCR1, pMB9 and pSC1. The transformed host may be eukaryotic or prokaryotic, preferably, a prokaryotic

host.

As used herein, the term "pharmaceutically acceptable" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the hosts to which it is administered.

- 5 As used herein, the term "prophylactic or therapeutic" treatment refers to administration to the host of the lymphokine(s) or cytotoxin(s) and biological modifier(s) either before or after onset of the biological damage to the host. If the lymphokine(s) or cytotoxin(s) and biological modifier(s) are administered prior to exposure to the agent causing the biological damage, the treatment is prophylactic (i.e., it protects the host against the damage), whereas if it is administered after exposure to the agent causing the damage, the treatment is therapeutic (i.e., it alleviates the existing damage). The scheduling and dosing will depend, e.g., on the type of host, disease, lymphokine or cytotoxin, and biological modifier. If the biological damage is caused by infection, the doses are preferably administered from 18 hours before infection for prophylactic treatment and in early phase of infection for therapeutic treatment, up to 18 hours after infection in later phase of infection for therapeutic treatment.
- 10 15 If the biological damage is cancer, the treatment is not considered therapeutic if after treatment a tumor appears or if an existing tumor burden is not eliminated or decreased. The effect of the doses will diminish with time, but for humans the dose may be repeated for months or even years. Prophylactic treatment of cancer refers to administration after the patient has been treated for cancer, to prevent reoccurrence of the cancer.
- 20 25 30 As used herein, the term "biological damage to the host caused by free radical generation" refers to any cellular, tissue or other damage to body parts or functions sustained by the host as a result of free radicals being produced in the body of the host. The free radicals may cause directly mobilization of the arachidonic acid metabolic pathways or may cause lipid peroxidation that mobilizes arachidonic acid. These radicals may be produced as a mechanism for killing cells. Examples by which such damage may be caused include hyperthermia, which may occur during cancer treatment as when the temperature of the tumor is increased via local or general microwave irradiation, damage caused by chemotherapeutic agents (chemotherapy), radiation therapy, or high oxygen tension that produce radicals to kill cells, and infection. Also, treated tumor cells may help propagate radical damage. An example of high oxygen tension is the condition that occurs when premature babies are exposed to high pressure oxygen, resulting in retinal and lung disease. Other conditions that represent damage caused by free radical generation may also be envisioned and fall within this definition.

The term "cancer" as used in the above definition refers to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer and gastrointestinal or stomach cancer. Preferably, the cancer is colon cancer, melanoma, renal cell cancer, sarcoma, lung cancer, adenocarcinoma, or breast cancer.

The term "infection" as used in the above definition refers to any kind of pathogenic disease, including those caused by bacteria, fungi, viruses, protozoa or parasites. Examples of bacterial infections include P. aeruginosa, E. coli, tetanus, Mycobacterium species, Streptococcal strains, diphtheria and Salmonella species. Examples of fungal infections include cryptococcosis, histoplasmosis, and other infections due to Candida species. Examples of viral infections include Hepatitis A, recurrent Herpes Simplex, AIDS, Herpes Zoster, influenza, and rhinoviruses. Preferably, the infection is bacterial, more preferably Gram-negative infection, and most preferably P. aeruginosa and E. coli infection.

As used herein, the term "biological modifier" refers to one of two compounds: a free radical scavenger or a metabolic inhibitor. The term "free radical scavenger" refers to any compound or substance that protects a mammalian host against biological damage caused by free radical generation. This definition includes those agents that act through direct radical scavenging as well as those that act by altering the radical scavenging ability of the host and/or tumor. Either mechanism will affect the response of the host to the lymphokine or cytotoxin. Such free radical scavengers may be radiation protectors, and include such compounds as, for example, uric acid, buthionine sulfoximine, diethyl maleate, vitamin E, vitamin C, cysteine such as N-acetyl cysteine, or glutathione, metronidazole, and a retinoid such as, e.g., vitamin A. While diethyl maleate may increase the toxicity of the lymphokine or cytotoxin, it also reduces glutathione preferentially in tumor versus host and therefore should lead to a higher therapeutic index. Any combination of these modifiers may be employed. Most preferably, the free radical scavenger employed herein for humans is buthionine sulphoximine, vitamin C, vitamin E, or N-acetyl cysteine [the latter having the tradename Mucomyst (Mead Johnson)]. See the 1987 Physicians' Desk reference, ed. 41, Barnhart, pub., Oradell, N.J: Medical Economics Company, Inc. Uric acid causes gout in humans so it may be tolerated in lesser amounts; it occurs naturally in human plasma at about 300  $\mu$ M; it is expected that about 600-1500

$\mu\text{M}$  might be tolerated in humans.

The second type of biological modifier, a "metabolic inhibitor," refers to a compound or substance that blocks or inhibits the cyclooxygenase and/or lipoxygenase metabolic pathways, of the arachidonic acid cascade, wherein phospholipids are converted to arachidonic acid, by phospholipase A2 or C, and the cascade, wherein phospholipids may proceed by either metabolic pathway. Such blockage or inhibition may be of an enzyme that catalyzes one or both pathways, of a cell type that contains the enzyme, or of one or more of the natural products of the pathways. Reduction in the number of those leukotrienes, hydroperoxy-eicosatetraenoic acid, hydroxy- and dihydroxy-eicosatetraenoic acids, prostaglandins, thromboxanes, and/or prostacyclins that lead to biological damage as defined herein indicates metabolic inhibition.

Examples of metabolic inhibitors include aspirin, indomethacin, ibuprofen, nordihydroguaiaretic acid (4,4'-[2,3-dimethyl-1,4-butanediyl]-bis[1,2-benzenediol]) (NDGA), cis-8,11,14-eicosatrien-5-ynoic acid (ETYA), and synthetic (as opposed to natural) prostaglandins and/or leukotrienes that block the effects of (ETYA), and synthetic (as opposed to natural) prostaglandins and/or leukotrienes that block the effects of (ETYA), and synthetic (as opposed to natural) prostaglandins and/or leukotrienes that block the effects of (ETYA). Aspirin, in the natural metabolic products at the production level rather than at the enzyme level. Aspirin, indomethacin, ibuprofen, and ETYA block the cyclooxygenase pathway, thereby inhibiting the production of natural prostaglandins, thromboxanes and prostacyclins. At higher concentrations, indomethacin also blocks phospholipase A<sub>2</sub>. NDGA and ETYA block the lipoxygenase pathway, thereby inhibiting the production of natural hydroperoxy-eicosatetraenoic acid, leukotrienes, and hydroxy- and dihydroxy-eicosatetraenoic acids. The preferred metabolic inhibitors herein for use with TNF are those selected from aspirin, indomethacin, and ibuprofen. Indomethacin is a non-steroidal antirheumatic agent with local antiinflammatory activity. Ibuprofen is a substitute for aspirin. Both drugs are referred to in the 1987 Physician's Desk Reference, supra.

As used herein, the term "pharmacologically effective amounts" as applied to the lymphokine or cytotoxin and the biological modifier refers to the amount of each component in the mixture or administered to the host that results in an increase in the therapeutic index of the host. The "therapeutic index" can be defined for purposes herein in terms of efficacy (extent of tumor or infection reduction or other cure) and in terms of toxicity to the host.

For non-human hosts, if the efficacy increases at least 50% over the efficacy using an excipient control (e.g., phosphate buffered saline) and the ratio of mean body weight at the end of the evaluation period for efficacy response to mean body weight at the start of treatment is at least 0.90 (i.e., no greater than 10% body weight loss), the therapeutic index has increased. The ratio of mean body weights indicates the extent of toxicity, with a value of 1 indicating no toxicity. For non-human hosts being treated for cancer, the extent of efficacy achieved may be measured by the ratio of mean tumor volume at the end of the evaluation period for efficacy response to mean tumor volume at the start of treatment. A reduction in the ratio of at least 50% of treated over excipient control indicates increased efficacy. The most preferred doses, schedules, and types of biological modifiers are those that achieve a mean tumor volume ratio of between 0 and 5, with a value of 0 being optimum and indicating a cure.

For human hosts, if the efficacy increases at least 50% upon treatment with the lymphokine/cytotoxin and biological modifiers and the toxicity is acceptable, i.e., no more than fever, chills, and/or general malaise, the therapeutic index has increased. For human hosts being treated for cancer, the extent of efficacy is generally ascertained in the clinic by measuring the perpendicular diameters of the products of all measured disease. A partial response occurs when the tumor shrinks by at least 50% in the sum of the products of the perpendicular diameters of all measured disease. For example, if a tumor having perpendicular diameters of 10 and 10 shrinks to perpendicular diameters of 8 and 8, the tumor has only shrunk from 100 to 64, which is not a 50% reduction and is not a partial response. If the tumor of 10 and 10 shrinks to 7 and 7, however, this is a partial response because it has shrunk from 100 to 49, more than 50%.

The use of this invention involves the production of a pharmaceutical composition which can be administered to a mammalian host, preferably a human host with pharmacologically effective amounts of one or more lymphokines or cytotoxins and one or more biological modifiers. The lymphokine(s), cytotoxin(s) and biological modifier(s) may be combined *in vitro* before administration or separately administered to the host, in either order or concurrently or simultaneously, with any administration of lymphokine/ cytotoxin generally taking place up to 24 hours after the administration of biological modifier, and with any administration of biological modifier taking place up to about one hour after the administration of lymphokine/cytotoxin. Preferably the biological modifier is added prior to adding or concurrently with the lymphokine or cytotoxin.

The administration(s) may take place by any suitable technique, including oral, subcutaneous and parenteral administration, preferably parenteral or oral. Examples of parenteral administration include intravenous, intraarterial, intramuscular, and intraperitoneal, with intraperitoneal and intravenous being

preferred.

The dose and dosage regimen will depend mainly on whether the lymphokine(s) or cytotoxin(s) and biological modifier(s) are being administered for therapeutic or prophylactic purposes, separately or as a mixture, the type of biological damage and host, the history of the host, the type of lymphokine or cytotoxin, and the type of biological modifier employed. The amount must be effective to achieve an enhanced therapeutic index as defined above. It is noted that humans are treated longer than the mice and rats exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses may be single doses or multiple doses over a period of several days, but single doses are preferred. For purposes herein, a protection level of at least 50% means that at least 50% of the treated hosts exhibit improvement against the infection, including but not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms.

Generally, for cancer, the dosage amount must be effective to achieve some tumor reduction or augmentation of lymphokineactivated killer (LAK) cell activity. LAK cells are lymphoid cells that can lyse fresh, noncultured, natural-killer-cell-resistant tumor cells but not normal cells. The doses may be single doses or multiple doses. If multiple doses are employed, as preferred, the frequency of administration will depend, for example, on the type of host and type of cancer, dosage amounts, etc. For some types of cancers or cancer lines, daily administration may be effective, whereas for others, administration every other day or every third day may be effective, but daily administration ineffective. The practitioner will be able to ascertain upon routine experimentation which route of administration and frequency of administration are most effective in any particular case.

The dosage amounts for cancer which appear to be most effective herein are those that result in regression in size of the tumor or complete disappearance or non-reappearance of the tumor, and are not toxic or are acceptably toxic to the host patient. Generally, such conditions as fever, chills and general malaise are considered acceptable. In addition, the dose of biological modifier cannot be so large as to inhibit the anti-tumor activity of the lymphokine or cytotoxin. These optimum dose levels will depend on many factors, for example, on the type of host, cancer, route, schedule and sequence of administration, existing tumor burden, the type of lymphokine or cytotoxin and biological modifier, and the definition of toxicity. Toxicity may be defined by the extent and type of side effects in human hosts, with fever, chills and general malaise considered acceptable toxicity for the study herein, or by the amount of body weight loss or by death in non-human hosts after a certain period of time, as defined above for the therapeutic index.

If TNF- $\alpha$  is employed as the lymphokine or cytotoxin, the dosage level thereof in humans is generally at least 0.24  $\mu\text{g}/\text{kg}$  patient weight, and in mice is at least 25  $\mu\text{g}/\text{kg}$ . As a general rule, the amount of TNF- $\alpha$  administered to humans is the approximate or exact number that is used for mice, but the units are  $\mu\text{g}/\text{m}^2$  rather than  $\mu\text{g}/\text{kg}$ . Preferably, TNF- $\alpha$  is administered to humans in an amount of about 25 to 100  $\mu\text{g}/\text{m}^2$  when buthionine sulphoximine is administered in a minimum effective radical scavenging concentration before and/or during the course of administration of the TNF- $\alpha$ . Smith et al., Proceedings of AACR, 28 - (March 1987), p. 440 discloses that in beagle dogs fifteen doses of 100 mg/kg/dose of BSO orally every eight hours is relatively non-toxic, whereas 400-800 mg/kg of BSO is toxic under those conditions of administration. Preferably, TNF- $\alpha$  is administered to humans in an amount of 125  $\mu\text{g}/\text{m}^2$  when vitamin C is administered in a minimum effective radical scavenging concentration prior to administration of the TNF- $\alpha$ . Preferably, TNF- $\alpha$  is administered to humans in an amount of 25-100  $\mu\text{g}/\text{m}^2$  when aspirin is administered in an amount of about 15-30 mg/kg of patient weight prior (e.g., 1-4 hours) to administration of TNF- $\alpha$ . Preferably, TNF- $\alpha$  is administered to humans in an amount of about 50-200  $\mu\text{g}/\text{m}^2$  when indomethacin is administered in an amount of about 25-50 mg prior to administration of the TNF- $\alpha$ . Preferably, TNF- $\alpha$  is administered to humans in an amount of about 150-250  $\mu\text{g}/\text{m}^2$  host when ibuprofen is administered in an amount of about 400 to 600 mg, when ibuprofen is administered prior to administration of TNF- $\alpha$ . Preferably, TNF- $\alpha$  is administered to humans in an amount of about 200-400  $\mu\text{g}/\text{m}^2$  when N-acetyl cysteine is administered in an amount equivalent to 250-1000 mg/kg of rat weight prior to the administration of TNF- $\alpha$ . The practitioner will be able to determine optimum dosage levels and scheduling when the host, lymphokine/cytotoxin and biological modifier are varied.

If IL-2 is employed as the lymphokine or cytokine, the dosage level thereof in humans is generally at least about  $3 \times 10^6$  units/ $\text{m}^2/\text{day}$  and in mice is at least about 5-10 mg/kg. Preferably, the IL-2 is administered to humans in an amount of at least  $3 \times 10^6$  units/ $\text{m}^2/\text{day}$  when vitamin C, vitamin E, aspirin, N-acetyl cysteine, ibuprofen or indomethacin is administered prior to administration of the IL-2.

The dosage level of CSF-1 in humans has not yet been determined, but in mice it may generally be about 50 mg/kg (at 100-150 mg/kg the mice being to die).

The typical dosage level of interferon (especially INF- $\beta$ ) in humans ranges from about 100 units to one

billion units/m<sup>2</sup>. Preferably, IFN- $\beta$  is administered to humans in an amount of at least 1000 units/m<sup>2</sup> when vitamin C, vitamin E, aspirin, N-acetyl cysteine, ibuprofen, or indomethacin is administered prior to administration of the IFN- $\beta$ .

For parenteral administration the lymphokine(s) will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable carrier medium which is inherently non-toxic and non-therapeutic or non-prophylactic. Examples of such vehicles include water, saline, Ringer's solution, dextrose solution, mannitol, and normal serum albumin. Non-aqueous vehicles such as fixed oils, propylene glycol and ethyl oleate may also be used. The carrier medium may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The lymphokine(s)/cytotoxins and biological modifiers will typically be formulated in such carriers at a concentration of about 0.1 mg/ml to 100 mg/ml of each, preferably 0.2 to 1 mg/ml of each.

Alternatively, if the lymphokine is IL-2, it may be made into a, sterile, stable lyophilized formulation in which the purified IL-2 is admixed with a water-soluble carrier such as mannitol, which provides bulk, and a sufficient amount of sodium dodecyl sulfate to ensure the solubility of the recombinant IL-2 in water. The formulation is suitable for reconstitution in aqueous injections for parenteral administration and it is stable and well-tolerated in human patients. The formulation method is more completely described in U.S. Patent No. 4,804,377. Alternatively, the IL-2 may be refolded using guanidine to obtain a more soluble product.

Guanidine may be used to solubilize the IL-2 particle paste, in a process incorporating the steps of:

- (a) disrupting the cell membrane of the microorganism;
- (b) separating water-insoluble, IL-2-containing material from the disruptate;
- (c) mixing the insoluble IL-2-containing material of step (b) at a pH of about 7 to about 9 with an aqueous solution of a reducing agent and a chaotropic agent whereby the IL-2 in the insoluble material is dissolved and denatured;
- (d) separating the IL-2-containing solution of step (c) from the undissolved portion of the insoluble material;
- (e) removing the reducing agent from the separated IL-2-containing solution;
- (f) oxidizing the IL-2 in the solution while maintaining the concentration of chaotropic agent at a strongly denaturing concentration, whereby the natural disulfide bridge of IL-2 is formed;
- (g) after the oxidation of step (f) is complete, diluting the solution to reduce the concentration of chaotropic agent in the solution to a level at which the oxidized IL-2 is permitted to renature and a precipitate forms;
- (h) separating the precipitate from the solution to provide a supernatant;
- (i) purifying the oxidized IL-2 in the supernatant by (1) reverse-phase high performance liquid chromatography followed by dissolution of the precipitate resulting from the chromatography in a solution of chaotropic agent and removal of the chaotropic agent from the solution, or (2) hydrophobic interaction chromatography followed by ion exchange chromatography; and
- (j) recovering a purified oxidized, soluble, heterologous human IL-2 composition having an IL-2 content of at least about 95% as determined by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis, a solubility in phosphate buffered saline of at least about 5 mg IL-2 per ml, a specific activity of at least about  $1 \times 10^6$  units/mg as determined by HT-2 cell proliferation assay, and an endotoxin content of less than about 0.1 nanograms per mg of IL-2.

In another embodiment, the guanidine may be used after the HPLC step, in a process described below. Briefly, the rIL-2 is separated from the bulk of the cellular components of the transformed microorganism hosts containing the rIL-2, the rIL-2 is solubilized in a reduced form, oxidized, purified to clinically acceptable purity and endotoxin levels, and denatured by placing the rIL-2 in a solution of a chaotropic agent. Thereafter, the solids are removed from the solution and the rIL-2 is renatured from the solution.

Preferably, the solution of a chaotropic agent is a 4 to 8 M aqueous guanidine hydrochloride solution.

In yet another alternative, the IL-2 may be administered in an adoptive immunotherapy method, together with isolated, lymphokine-activated lymphocytes, in a pharmaceutically acceptable carrier, where the lymphocytes have antitumor activity when administered with the IL-2 to humans suffering from the tumor. This method is described more fully in U.S. Patent No. 4,690,915 issued September 1, 1987, and by S. Rosenberg et al., *New England Journal of Medicine* (1985), 313:1485-1492. In another alternative, described in S. Rosenberg et al., *Science*, 233:1318-1321 (1986), tumor-infiltrating lymphocytes (TIL) expanded in IL-2 may be adoptively transferred for the therapeutic treatment, particularly in combination with cyclophosphamide. The TIL approach may also be used herein.

As mentioned above, the recombinant lymphokine employed herein may be any lymphokine, obtained from tissue cultures or by recombinant techniques, and from any mammalian source such as, e.g., mouse, rat, rabbit, primate, pig, and human. Preferably the lymphokine is derived from a human source and more

preferably is a human recombinant lymphokine. Most preferably the lymphokine is recombinant human IL-2 or TNF alone or in combination with recombinant TNF or IL-2, respectively.

The recombinant IL-2 may be obtained as described by Taniguchi et al., Nature, 302:305-310 (1983) and Devos, Nucleic Acids Research, 11:4307-4323 (1983) by cloning the native human IL-2 gene and expressing it in transformed microorganisms. It may also be an IL-2 mutein as described in U.S. Patent No. 4,518,584, in which the cysteine normally occurring at position 125 of the wild-type or native molecule has been replaced by a neutral amino acid such as serine or alanine, or an IL-2 mutein as described in EP-A-0 200 280, published November 5, 1986, in which the methionine normally occurring at position 104 of the wild-type or native molecule has been replaced by a neutral amino acid such as alanine.

Preferably, the IL-2 is an unglycosylated protein which is produced by a microorganism which has been transformed with the human cDNA sequence or a modified human cDNA sequence of IL-2 which encodes a protein with an amino acid sequence at least substantially identical to the amino acid sequence of native human IL-2, including the disulfide bond of the cysteines at positions 58 and 105, and has biological activity which is common to native human IL-2. Substantial identity of amino acid sequences means the sequences are identical or differ by one or more amino acid alterations (deletions, additions, substitutions) which do not cause an adverse functional dissimilarity between the synthetic protein and native human IL-2. Examples of IL-2 proteins with such properties include those described by Taniguchi et al., Nature (1983), 302:305-310; Devos, Nucleic Acids Research (1983), 11:4307-4323; and by EP-A-0 091 539 and 0 088 195; in U.S. Patent 4,518,584, supra, and in EP-A-0 200 280, supra. Most preferably, the IL-2 is the desal<sub>α</sub>-IL-2<sub>ser125</sub> mutein in which the initial terminal alanine is deleted and the cysteine at position 125 is replaced by a serine residue.

The IL-2 may be produced and purified to clinical purity by the method described and claimed in U.S. Patent No. 4,569,790, issued February 11, 1986. In addition, the IL-2 may be recovered from refractile bodies using sucrose as described in EP-A-0 206 828, published December 30, 1986. The IL-2 may also be modified by derivatization with polyethylene glycol or a polyoxyethylated polyol, as described in PCT 87/00056, published January 15, 1987.

The human TNF- $\alpha$  herein may be obtained in recombinant form as described by Pennica et al., Nature (1984) 312:724-729; Yamada et al., J. Biotechnology, (1985) 3:141-153; Wang et al., Science, (1985) 228:149-154; Shirai et al., Nature (London), (1985) 313:803-806; EP-A-0 155 549, published September 29, 1985; EP-A-0 158 286, published October 16, 1985; EP-A-0 168 214, published January 15, 1986; and PCT US85/01921, published April 24, 1986. Recombinant rabbit TNF- $\alpha$  may be obtained as described in EP-A-0 146 026, published June 26, 1985 and EP-A-0 148 311, published July 17, 1985.

The cloning of human TNF- $\alpha$  having 151 and 155 amino acids (2 and 6 less than the native form) is disclosed in EP-A-0 155 549, published September 25, 1985 (Dainippon Pharmaceutical Co., Ltd.), and human TNF- $\alpha$  having 155 amino acids is disclosed in EP-A-0 158 286, published October 16, 1985 (Asahi Kasei Kogyo Kabushiki Kaisha) and corresponding GB 2,158,829A, published November 20, 1985. The cloning of mature TNF- $\alpha$  (157 amino acids) and various modified forms (muteins) thereof is disclosed in EP-A-0 168 214, published January 15, 1986 (Genentech), and U.S. Patent Nos. 4,677,064 issued June 30, 1987 and 4,677,063 issued June 30, 1987 (both Cetus Corporation).

Preferably, the TNF- $\alpha$  herein is a human TNF mutein wherein one or more of the first eight amino acid residues, preferably either the first four or the first eight, have been deleted, using the procedure described in U.S. Patent No. 4,677,064, supra, or the TNF- $\alpha$  is a cysteine-depleted mutein described in U.S. Patent No. 4,677,063 issued June 30, 1987, supra, and in U.S. Patent No. 4,518,584, supra. The TNF may be purified by the method described in EP-A-0 220 986, published May 6, 1987.

The precise chemical structure of the TNF- $\alpha$  herein will depend on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular form of TNF- $\alpha$  may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their bioactivity when placed in suitable environmental conditions are included in the definition of the TNF- $\alpha$  herein. Further, the primary amino acid sequence of the TNF- $\alpha$  may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate or acetyl groups, more commonly by conjugation with saccharides. Certain aspects of such augmentation are accomplished through posttranslational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition of TNF- $\alpha$  herein so long as the bioactivity of the TNF- $\alpha$  is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the bioactivity by either enhancing or diminishing the activity of the TNF- $\alpha$  in the various assays.

In one formulation, the TNF- $\alpha$  may be reacted with a homopolymer or copolymer of polyethylene glycol or a polyoxyethylated polyol, provided that the polymer is soluble in water at room temperature. The

polymer is reacted first with a coupling agent having terminal groups reactive with both the free amino or thiol groups of the protein and the hydroxyl group of the polymer. Examples of such coupling agents include hydroxynitrobenzene sulfonic ester, cyanuric acid chloride and N-hydroxysuccinimide. The TNF- $\alpha$  is then formulated directly with the water-soluble carrier and buffer as described above, and the formulation may be lyophilized and the lyophilized mixture reconstituted as described above.

5 Recombinant IFN- $\gamma$  may be obtained as described by Gray et al., *Nature*, 295:503 (1982).

The various aspects of the invention are further described by the following examples, which are not intended to limit the invention in any manner. In these examples all parts for solids are by weight and all percentages for liquids and gases are by volume, unless otherwise noted, and all temperatures are given in 10 degrees Celsius.

EXAMPLE 1 - Use of Uric Acid

A. General Treatment

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Mice

Female Balb/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) that were all 6-8 weeks old were employed in the in vivo tests. Animals were weight matched at  $20 \pm 3$  g, randomized at five per 20 cage, and ear-notched. All animals were held in quarantine observation for seven days after arrival, maintained in microisolator cages (Lab Products, Inc.), and fed standard laboratory diets and drinking water ad lib.

TNF- $\alpha$

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A mutein of human TNF- $\alpha$  having the first eight amino acids deleted from the N-terminus was prepared as described in U.S. Patent No. 4,677,064 issued June 30, 1987 and Wang et al., *Science* (1985) 238:149-153. Briefly, TNF was induced from HL-60 cells and purified and sequenced. Then an intronless sequence encoding human TNF was prepared by producing enriched mRNA, constructing a cDNA library, selecting a probe and probing the library to recover the sequence. Then an ATG start codon was introduced immediately preceding the GTC sequence encoding N-terminal valine of the mature protein by site-directed mutagenesis. Clones were selected and strands ligated into expression vectors to obtain prokaryotic expression of the mutein. The mutein was then purified by column purification using any standard purification technique and recovered in the purification buffer. The mutein was produced as a lyophilized powder in sterile vials, reconstituted and suspended using sterile phosphate buffered saline within four days prior to use, and stored, if at all, at  $4^{\circ}\text{C}$ . The TNF contained less than 0.001 to 0.006 ng endotoxin/mg protein depending on production lot.

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Free Radical Scavenger

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The free radical scavenger employed herein was commercially available uric acid. (Sigma).

Cancer Cell Line and Tumor Injections

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The target cells employed were a methylcholanthrene-induced murine fibrosarcoma (Meth-A) (Balb/c) obtained as an ascites-passed tumor from Dr. Lloyd Old, Memorial Sloan-Kettering Cancer Center, New York, New York, frozen as stock, and passed at least twice in ascites prior to use. These cells were implanted subcutaneously in the suprascapular area of the mouse host.

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B. Results

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Table I indicates the results obtained when the TNF mutein alone, uric acid alone, and various combination injections of the TNF mutein and uric acid were administered intravenously to five mice per group (2-3 repeats) beginning seven days after tumor implantation and every third day three times, with final measurements made on the 14th day. The excipient control was injected with PBS.

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The uric acid dose was slightly below the maximum concentration attainable without decreasing animal weight, determined in a preliminary toxicity study, and was given immediately prior to the TNF inoculation.

The TNF doses were chosen to cover the ranges of 50, 100, 150, 200 and 250  $\mu\text{g}/\text{kg}$  of mice weight

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(the mice weighing approximately 20 g each). The uric acid doses were 25 mg/kg of mouse weight whenever uric acid was administered.

TABLE I

Group	$\Delta BW^a$	Deaths	$\Delta TW^b$	Cures <sup>c</sup>
Excipient controls	1.36 ± .13	1/15*	60.4 ± 23.9	0/14
Uric acid	1.29 ± .07	1/15*	51.7 ± 17.7	0/14
TNF (50 µg/kg)	1.1 ± .14	1/15	7.9 ± 6.2	1/14
TNF (50 µg/kg) + uric acid	1.05 ± .02	1/15*	10.5 ± 5.9	0/14
TNF (100 µg/kg)	1.0 ± .02	1/10	1.4 ± 0.6	0/9
TNF (100 µg/kg) + uric acid	1.07 ± .12	1/10*	16.4 ± 22	0/9
TNF (150 µg/kg)	1.04	6/10	0.42	0/4
TNF (150 µg/kg) + uric acid	1.02 ± .07	1/10*	8.7 ± 7.3	0/9
TNF (200 µg/kg)	1.01 ± .03	5/10	0.48	3/5
TNF (200 µg/kg) + uric acid	1.04 ± .02	3/10	10.6 ± 3.1	0/7
TNF (250 µg/kg)	—	15/15	—	—
TNF (250 µg/kg) + uric acid	1.05 ± .07	8/15	1.21 ± 0.5	0/7

\*  $\Delta BW$  = change in body weight as measured by the ratio of mean body weight (in g) at 14 days after treatment to mean body weight (in g) at the start of treatment.

<sup>b</sup>  $\Delta TW$  = change in tumor volumes as measured by the ratio of mean tumor volume (in mm<sup>3</sup>) at 14 days after treatment to mean tumor volume (in mm<sup>3</sup>) at the start of treatment.

<sup>c</sup> Cures =  $\Delta TW=0$ , or no visible tumor after 21 days from start of treatment.

\* = "Tumor death", not toxic death.

The results show that combined treatment of the mice resulted in a decrease in toxicity over both PBS control and TNF alone as measured by host death, and an increase in efficacy as measured by comparing the  $\Delta TW$  of the PBS control and the  $\Delta TW$  of the combinations. Therefore, the therapeutic index as defined herein has been enhanced.

Uric acid has been postulated by Ames et al., *supra*, as a major protective agent against damage by reactive oxygen species and lipid peroxidation present in primates, but not rodents. The results of these experiments indicate that reactive oxygen radicals and/or lipid peroxide-like products play a role in the mechanism of action of TNF.

The practitioner can predict that these results likely would apply to humans based on the expected correlation between the TNF dose-related anti-tumor effect in animals and humans. The preclinical response of TNF alone correlated with a clinical response of TNF to colon cancer.

#### EXAMPLE 2 - Use of Vitamin C (Ascorbic Acid)

Female Balb/c mice were implanted subcutaneously with Meth-A tumor cells as described in Example 1.

Table II indicates the results obtained when either vitamin C or the TNF mutoein of Example 1 alone, or various combinations of vitamin C immediately followed by the TNF mutoein were administered intravenously to groups of 5 mice beginning seven days after tumor implantation and continuing every third day for three injections, with measurements taken on the fourteenth day following the initiation of treatment.

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TABLE II

	TNF $\mu\text{g}/\text{kg}$	Vit.C $\text{mg}/\text{kg}$	% Control $\Delta\text{BW}^a$	Deaths	% Control $\Delta\text{TW}^b$	"Cures" <sup>c</sup>
5	125	0	81	1/10	1.5	4/9
	125	7	92	0/5	—	5/5
	125	35	86	0/5	10.0	0/5
	125	70	83	2/5	12.0	0/5
	0	0	100	0/5	100	0/5
10	0	7	92	0/5	76	0/5
	0	35	100	0/5	88	0/5
	0	70	96	0/5	74	0/5

<sup>a</sup>  $\Delta\text{BW}$  = change in body weight as measured by the ratio of mean body weight (in g) at 14 days after treatment to mean body weight (in g) at the start of treatment; % control is the normalized value relative to the control groups.

<sup>b</sup>  $\Delta\text{TW}$  = change in tumor volumes as measured by the ratio of mean tumor volume (in  $\text{mm}^3$ ) at 14 days after treatment to mean tumor volume (in  $\text{mm}^3$ ) at the start of treatment; % control is the normalized value relative to the control groups.

<sup>c</sup> Cures =  $\Delta\text{TW}=0$ , or no visible tumor after 21 days from start of treatment.

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Vitamin C functions as an aqueous compartment radical scavenger, as well as in collagen stability. The results indicate that treatment with vitamin C and TNF at 125  $\mu\text{g}/\text{kg}$  TNF dose resulted in fewer cures and little alteration in toxicity, but enhancement of efficacy over the PBS control at the two higher 25 vitamin C doses. At high doses of TNF (250  $\mu\text{g}/\text{kg}$ ), buffering with vitamin C was not possible. Vitamin C alone was non-toxic at the three doses tested. As rodents have endogenous ascorbic acid, there may be regulatory mechanisms that influence exogenous administration. However, vitamin C appears to improve the therapeutic index significantly at the 7 mg/kg dose.

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### EXAMPLE 3 - Use of Buthionine Sulphoximine (BSO)

Female Balb/c mice were implanted subcutaneously with Meth-A tumor cells as described in Example 1. Treatment was initiated seven days after the implantation. A protocol was employed wherein BSO or the TNF murein of Example 1 alone, or BSO together with the TNF murein, were administered. BSO was administered intraperitoneally and TNF intravenously. The BSO was started 24 hours prior to TNF administration twice daily (6-8 hours between doses) for ten days and TNF was given with the second BSO dose every third day for three times. BSO excipient was used as a volume control in the TNF alone groups. PBS was used as a control. The protocol is illustrated below:

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(Days) -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

BSO	TNF	BSO	BSO	TNF	BSO	BSO	TNF	BSO	BSO						
BSO		BSO			BSO			BSO							

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Table III illustrates the results of this study, with the headings defined in the footnotes to Table II. The numbers in parentheses indicate the results from a repeat of the experiment.

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TABLE III

Inhibition of Glutathione Synthesis by Buthionine Sulfoximine Increased the Sensitivity of Meth-A tumor cells to TNF In Vivo <sup>a</sup>						
	Treatment Group	ΔBW <sup>b</sup>	Deaths	ΔTW <sup>c</sup>	Cures <sup>d</sup>	Dose Modification Factor <sup>e</sup>
	BSO (1.25 g/kg)	1.19	0/10	29.8	0/10	--
	TNF (25 µg/kg)	1.09	0/10	7.8	1/10	--
	TNF (25 µg/kg) + BSO	1.03	0/10	1.8 <sup>f</sup>	0/10	4.3
	TNF (50 µg/kg)	1.04	0/10	4.9	1/10	--
	TNF (50 µg/kg) + BSO	0.98	0/10	1.1 <sup>f</sup>	8/10	4.4
	TNF (100 µg/kg)	0.99	1/10	1.9	3/9	--
	TNF (100 µg/kg) + BSO	0.99	1/10	--	9/9	∞
	Controls (saline)	1.20	0/10	30.0	0/10	--

<sup>a</sup> Data are for two independent experiments: 5 mice/group<sup>b</sup> mean values of two experiments<sup>c</sup> mean values<sup>d</sup> "Cures" indicates no palpable tumor on day 14<sup>e</sup> "Dose modification factor" is the ratio of ΔTW of TNF alone/ΔTW of TNF + BSO<sup>f</sup> Significantly different from TNF alone group by Quade rank analysis of variance (p set at 0.05)

The results of these experiments indicate that the pretreatment with BSO increased the therapeutic index of the TNF at all doses of TNF tried; it increased the anti-tumor efficacy of TNF with little increase in toxicity, as illustrated in the column headed "Dose Modification Factor." The data further support the hypothesis that the in vivo mechanism of efficacy, and to some degree the toxicity, is related to free-radical production. Tumor cell sensitivity/resistance depends on the ability of the tumor cells to protect themselves from free radical damage.

The following Table IV illustrates the sensitivity of various human cell lines to TNF and the relationship of TNF sensitivity to glutathione (GSH) content in the cells. BSO is used to reduce preferentially the GSH level of Meth-A as compared to the host, thereby depleting the radical scavenging capacity of the tumor. All cell lines mentioned below are available from the American Type Culture Collection (ATCC) in Rockville, MD.

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TABLE IV

Intracellular Glutathione Levels Correlate With TNF Resistance In Vivo			
	Tumor Line	µM Total Glutathione Equivalents /10 <sup>6</sup> cells	% Tumor Growth Inhibition <sup>a</sup>
5	PAN-02 (mouse tumor)	484 ± 90	0
	HT29 (human colon tumor)	308 ± 131	9
10	P815 (mouse tumor)	305 ± 197	20
	P388 (mouse tumor)	280 ± 150	15
	B-16 (mouse tumor)	180 ± 56	60
15	L1210 (mouse tumor)	105 ± 37	74
	ME180 (human cervical carcinoma)	14.5 ± 4.6	ND <sup>b</sup>
	L929 (mouse tumor)	14.0 ± 0.7	ND <sup>b</sup>
	Meth A (mouse tumor)	9.1 ± 4.3	100

<sup>a</sup> Determined at the maximum tolerated dose of TNF at an endpoint defined as the control tumor increasing in volume at least 20-fold; for these models generally 17-28 days post-implantation. For the L1210, P388, P815, and B-16 tumor lines, mice were treated daily for 14 days i.p., at 250 µg/kg, one day following subcutaneous tumor inoculation. PAN-02 and HT29 were treated every third day, three times, i.v., at 150 or 100 µg/kg, respectively, starting 7 days after tumor inoculation. These conditions resulted in less than 10% body weight loss vs. controls, and were considered to be the maximal anti-tumor efficacy signal obtainable in these models without inducing non-specific tumor growth inhibition due to host toxicity.

<sup>b</sup> Not determined. Previously published data have shown that in vitro, the dose of TNF at which 50% of the animals die (TCID<sub>50</sub>) for ME180 = 50 µg/ml; L929 = 20 µg/ml [Creasey et al., Canc. Res., 47:145-149 (1987)].

#### EXAMPLE 4 - Use of Aspirin

Female Balb/c mice were treated by subcutaneous implantation of Meth-A tumor cells as described in Example 1. Five mice per group were employed. Treatment was initiated seven days after the implantation.

A protocol was employed wherein aspirin or the TNF mutein of Example 1 alone, or aspirin together with the TNF mutein, were administered intravenously. In the combination group, aspirin was administered daily for five days and each dose was followed 1-4 hours later by TNF. PBS was used as a control.

When 250 µg/kg TNF was administered after a single dose of 30 mg/kg aspirin or doses of aspirin once a day for five days, 9/10 aspirin-treated mice died within 48 hours of treatment with TNF. In contrast, aspirin alone was non-toxic, and TNF alone resulted in 1/5 mice dead.

The dosage of TNF was reduced to 25-150 µg/kg host weight, and the results are indicated in Table V, where final measurements were taken 14 days after treatment began. The headings are defined in the footnotes of Table I.

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TABLE V

Group	$\Delta BW$	Deaths	$\Delta TW$	"Cures"
Aspirin (30 mg/kg)	1.34	0/5	66.6	0/5
PBS	1.25	0/5	54.2	0/5
TNF (25 $\mu$ g/kg)	1.15	0/5	23.8	0/5
+ aspirin (30 mg/kg)	1.17	1/5	23.4	0/4
TNF (50 $\mu$ g/kg)	1.15	0/5	11.3	1/5
+ aspirin (30 mg/kg)	1.12	0/4	6.9	3/4
TNF (100 $\mu$ g/kg)	1.06	0/5	3.2	2/5
+ aspirin (30 mg/kg)	1.09	1/5	5.6	3/4
TNF (150 $\mu$ g/kg)	0.98	0/5	1.5	0/5
+ aspirin (30 mg/kg)	1.10	3/5	0.7	1/2

The results indicate that at the lower TNF doses, pretreatment with aspirin enhanced the therapeutic index of TNF (increased efficacy at the expense of a slight toxicity increase). In fact, the increase in efficacy over TNF alone as measured by  $\Delta TW$  was by a factor of about 2 at 50  $\mu$ g/kg TNF in the presence of 30 mg/kg aspirin, and, further, 3 out of 4 cures were obtained in the combination group compared to 1 out of 5 with TNF alone. At the 100  $\mu$ g/kg TNF level, a similar trend was seen, as 3 out of 4 cures were obtained in the combination group compared to 2 out of 5 cures in the TNF alone group. At 150  $\mu$ g/kg, the aspirin combination was more toxic with little change in efficacy.

#### EXAMPLE 5 - Use of Nordihydroguaiaretic Acid (NDGA), Aspirin, or the Combination with TNF

Female Balb/c mice were treated by subcutaneous implantation of Meth-A tumor cells as described in Example 1. Table VI Indicates the results obtained when NDGA (from Sigma Chemical Co.), aspirin, the combination of NDGA and aspirin, the TNF mutein of Example 1 alone, and various combinations of NDGA and aspirin followed by the TNF mutein were administered to groups of 5 mice beginning seven days after tumor implantation and continuing every third day three times. Details of the protocols are in the footnotes of Table VI. PBS was used as a control. Results were evaluated 14 days after initiation of treatment. The headings are defined in the footnotes of Table I.

TABLE VI

Group	$\Delta BW$	$\Delta TW$	Cures	Deaths
TNF <sup>a</sup>	1.04	2.0	0/5	0/5
+ aspirin <sup>b</sup>	1.08	3.8	0/5	0/5
+ NDGA <sup>c</sup>	1.04	3.1	0/5	0/5
+ aspirin + NDGA	1.03	0.7	0/5	0/5
Aspirin (30 mg/kg) <sup>d</sup>	1.2	60.3	0/5	0/5
NDGA (15.6 mg/kg) <sup>e</sup>	1.3	50.8	0/5	0/5 (1 tumor death d. 14)
Aspirin (30 mg/kg) + NDGA (15.6 mg/kg) <sup>f</sup>	1.4	47.8	0/5	1/5 (d. 5)
PBS	1.3	50.4	0/5	0/5

<sup>a</sup> = 100  $\mu$ g/kg i.v. every third day 3 times for all combinations.

<sup>b</sup> = 30 mg/kg iv. one hour prior to each TNF administration.

<sup>c</sup> = 312  $\mu$ g/20 g mouse (15.6 mg/kg) in propylene glycol i.p. 5 minutes prior to each TNF administration.

<sup>d</sup> = i.v. every third day 3 times.

<sup>e</sup> = i.p. every third day 3 times.

<sup>f</sup> = aspirin i.v. every third day 3 times; NDGA i.p. one hour after each aspirin administration

NDGA is known to inhibit the 5'-lipoxygenase pathway, which would be expected to inhibit leukotriene synthesis and, thereby, the toxicities associated with their production in the host. In addition, NDGA may function in a radical scavenging role in some cases, which role would be expected also to reduce the

toxicity of TNF. The exact mechanisms at work under the present conditions are not entirely known at this time, but evidence has been presented to suggest that either effect or both effects would be expected to reduce TNF toxicity.

5 EXAMPLE 6 - Use of Indomethacin, Ibuprofen or Aspirin with TNF

Female Balb/c mice were treated by subcutaneous implantation of Meth-A tumor cells as described in Example 1.

Table VII indicates the results obtained when aspirin, indomethacin, ibuprofen, the TNF mutein of Example 1 alone, and either aspirin, indomethacin or ibuprofen followed in two hours by the TNF mutein were administered to groups of 10 mice and of five mice beginning seven days after tumor implantation. Details of the protocols are referenced in the footnotes of Table VII. Results were evaluated 14 days after initiation of treatment for TGI, and at least 21 days, usually 28 days, for cure data. TGI is tumor growth inhibition, calculated as the % ratio of the weight of the treated tumor at day 14 to the weight of the tumor at day 14 of the controls, subtracted from 100. (For example, if treated volume is 30 and control volume is 1200, the ratio is 2.5%, and the % TGI is 97.5.) Cures are the same as defined in footnote c of Table I.

The results show the best dosage for mice of TNF for 3 mg/kg indomethacin to be about 50-200 µg/kg host weight (50-200 µg/m<sup>2</sup> for humans). The effects of ibuprofen in combination with TNF were approximately equal to those of aspirin in combination with TNF. The results confirm the best dose of TNF for 30 mg/kg host aspirin.

EXAMPLE 7 - Use of N-Acetyl Cysteine with TNF

CD rats purchased from Charles River Labs were injected with either 200 or 400 µg/kg of the TNF mutein described above in Example 1 intravenously once at 0 hours. In addition, CD rats were injected intravenously 24 hours and one hour prior to TNF treatment iv at 0 hours with N-acetyl cysteine (Sigma) at 250 and 100 mg/kg host. The data shown in Table VIII are the deaths within 24 hours/total rats treated. It can be seen that the administration of the N-acetyl cysteine reduces the toxicity of the TNF at all doses tested.

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TABLE VII  
Effects of Cyclooxygenase Inhibitors  
on TNF Toxicity and Efficacy

Dose Group	Toxic Deaths/ Total Treated	Percent Deaths	Day of Death	Percent TGI	Percent Cures/Survivors
TNF (50 µg/kg) <sup>a</sup>	0/40	0	-	75	30
+INDO <sup>b</sup>	0/25	0	-	94	68
+IBU <sup>b</sup>	2/25	8	10.5 (7-14)	72	35
+ASA <sup>c</sup>	0/30	0	-	86.5	35
TNF (100 µg/kg) <sup>d</sup>	4/35	11.4	1.5 (1-6)	91.6	65
+INDO <sup>b</sup>	1/25	4	1	95.3	92
+IBU <sup>b</sup>	4/25	16	2 (1-4)	87.7	62
+ASA <sup>e</sup>	2/25	8	1	93.3	60
TNF (200 µg/kg) <sup>d</sup>	26/35	74	1 (1-5)	96	75
+INDO <sup>b</sup>	14/25	56	1 (1-4)	99.5	91
+IBU <sup>b</sup>	21/25	84	1 (1-7)	99	100
+ASA <sup>f</sup>	34/40	85	1 (1-6)	96	67
TNF (300 µg/kg) <sup>a</sup>	33/40	82.5	1 (1-6)	100	67

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TABLE VII (Cont'd)

Dose Group	Toxic Deaths/ Total Treated	Percent Deaths	Day of Death	Percent TGI	Percent Cures/Survivors
+INDOb	24/25	96 (1-4)	1 (1-4)	100	100
+IBUb'	25/25	100	1	-	-
+ASA <sup>d</sup>	33/35	94.3 (1-5)	1 (1-5)	95	100

a TNF administered intravenously every third day for three times; 6 expts., with 2 expts. at 10 mice/grp. and 4 expts. at 5/mice grp.

b Indomethacin (3 mg/kg) administered ip, 2 hours before TNF every third day for three times; 3 expts., with 2 expts. at 10 mice/grp., and 1 expt. at 5 mice/grp.

b' Ibuprofen (20 mg/kg) administered ip, 2 hours before TNF every third day for three times.

c Aspirin (30 mg/kg) IV, 2 hours before TNF every third day for three times; 4 expts. with 2 expts. at 10 mice/grp. and 2 expts. at 5 mice/grp.

d 5 Expts., with 2 expts. at 10 mice/grp. and 3 expts. at 5 mice/grp.

e 3 Expts. with 2 expts. at 10 mice/grp. and 1 expt. at 5 mice/grp.

f 6 Expts. with 2 at 10 mice/grp. and 4 expts. at 5 mice/grp.

Endotoxin (LAL's): INDO = >0.12/<1.2 ng/ml (200  $\mu$ l dosing volume)  
 IBU = <0.2 ng/ml  
 ASA = <0.01 ng/ml

TABLE VIII

Deaths Within 24 Hours/Total Rats Treated			
	0 mg/kg N-acetyl cysteine	250 mg/kg N-acetyl cysteine	1000 mg/kg N-acetyl cysteine
200 µg/kg TNF	9/10	4/10	0/10
400 µg/kg TNF	8/10	4/10	0/10

The above effects are expected to be observed in lymphokines or cytotoxins other than TNF. For example, the biological modifiers may play a role in IL-2 mediated tumorcidal activity. There has been found a clear relationship between the amount of radical scavenging capacity of various tumor cells (total glutathione content) and their sensitivity to both TNF and IL-2. The following experiment illustrates that IL-2 may be as effective as TNF.

#### EXAMPLE 8 - Use of Uric Acid with IL-2

Female Balb/c mice were implanted subcutaneously with Meth-A tumor cells as described in Example

- An IL-2 mutein, designated des-ala<sub>1</sub>-IL2<sub>ser125</sub> (with no alanine at position 1 of the mature IL-2 molecule and a serine at position 125 of the mature IL-2 molecule), was prepared by the method described in U.S. Patent No. 4,518,584, supra, isolated from refractile bodies in accordance with the procedure described in EP-A-0 206 828, published December 30, 1986, and formulated as described in U.S. Patent No. 4,604,377 in sodium dodecyl sulfate. The designation des-ala<sub>1</sub>-IL2<sub>ser125</sub> indicates that the initial alanine of the mature native IL-2 sequence is missing and that the cysteine residue at position 125 of the mature native IL-2 sequence has been substituted with a serine.

Table IX indicates the results obtained when the IL-2 mutein described herein alone, uric acid alone, and various combination injections of the IL-2 mutein and uric acid were administered intravenously to five mice per group (2-3 repeats) beginning seven days after tumor implantation and once daily for five days, with the uric acid being administered immediately prior to (about 10 minutes) the IL-2 administration when both were administered. Final measurements were made on day 14. The excipient control was injected with PBS. The uric acid doses were all 25 mg/kg of mice weight. The headings are defined in the footnotes of Table I.

TABLE IX

Group	ΔBW	Deaths	ΔTW	Cures
Excipient controls	1.18	0/5	30.5	0/5
Uric acid	1.29	0/5	42.3	0/5
IL-2 (1.5 mg/kg)	1.08	0/5	11.6	0/5
IL-2 (1.5 mg/kg) + uric acid	1.13	0/5	18.4	0/5
IL-2 (5.0 mg/kg)	1.07	0/5	3.9	0/5
IL-2 (5.0 mg/kg) + uric acid	1.11	0/5	8.1	0/5

The results show that combined treatment of the mice resulted in a decrease in toxicity over the IL-2 control; however, the IL-2 was about 2-fold less efficacious when given with uric acid. This decrease is similar in magnitude to what was observed with lower TNF doses and uric acid. It is predicted that longer treatments and an increase in the dose of IL-2 will result in cures of the tumor. The preferred biological modifiers for human use are aspirin, vitamin C, vitamin E, ibuprofen, indomethacin, and N-acetyl cysteine. For humans, IL-2 is typically administered at a level of  $3 \times 10^6$  units/m<sup>2</sup> per day.

The present invention achieves the following goals: First, the ability of the host or tumor to scavenge radicals is reduced by the combination therapy, for example with BSO, thereby increasing the therapeutic index of the tumor over the host. Second, the biological damage at the host level caused by free radical production is blocked, e.g., by the plasma-mediated radical scavenger, uric acid. Third, the metabolism of the arachidonate cascade, which is influenced by and itself produces reactive radical species in lipid and aqueous compartments, can be modulated to increase the therapeutic index of the lymphokine or cytotoxin.

Effective amounts of the biological modifier can be determined for humans based on translations from mice data when human clinical trials are undertaken.

In summary, the present invention is seen to provide a combination of lymphokine or cytotoxin and biological modifier that results in an enhanced therapeutic index relative to the lymphokine or cytotoxin alone in a mammalian host.

**Claims**

**Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE**

10. 1. A composition suitable for administration to mammalian hosts for therapeutic or prophylactic treatment of biological damage to the host caused by free radical generation, which composition comprises a mixture, in pharmacologically effective amounts, of at least one lymphokine or cytotoxin from a mammalian species and at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor.
15. 2. The composition according to claim 1 wherein the lymphokine or cytotoxin is interleukin-2, interferon- $\beta$ , tumor necrosis factor, or colony stimulating factor-1.
20. 3. The composition according to claim 1 or 2 wherein the lymphokine or cytotoxin is tumor necrosis factor-alpha.
25. 4. The composition according to any one of claims 1 - 3 wherein the biological modifier is selected from uric acid, buthionine sulphoximine, vitamin E, vitamin C, N-acetyl cysteine, a retinoid, glutathione, metronidazole, aspirin, indomethacin, ibuprofen, nordihydroguaiaretic acid, cis-8,11,14-eicosatrien-5-ynoic acid, synthetic prostaglandins, synthetic leukotrienes, and combinations of one or more of these modifiers.
30. 5. The composition according to any one of claims 1 - 2 or 4 wherein the lymphokine is interleukin-2.
35. 6. The composition according to claim 5 wherein the biological modifier is uric acid and the interleukin-2 is a desalanyl mutein with a serine residue at position 125 of the native interleukin-2 molecule.
40. 7. Use of pharmacologically effective amounts of at least one lymphokine or cytotoxin from a mammalian species and at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor for the production of a pharmaceutical composition for the therapeutic or prophylactic treatment of biological damage to a mammalian host caused by free radical generation.
45. 8. The use of at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor for the production of a pharmaceutical composition for enhancing the therapeutic index of at least one lymphokine or cytotoxin from a mammalian species in the therapeutic or prophylactic treatment of biological damage to a mammalian host caused by free radical generation.
9. Use according to claim 7 or 8 wherein the lymphokine or cytotoxin is selected from an interleukin, an interferon, a tumor necrosis factor, or a colony stimulating factor.
45. 10. Use according to any one of claims 7 - 9 wherein the biological damage is cancer, infection, or damage caused by high oxygen tension, radiotherapy or chemotherapy.

**Claims for the following Contracting States : ES, GR**

50. 1. A process for the preparation of a composition suitable for administration to mammalian hosts for therapeutic or prophylactic treatment of biological damage to the host caused by free radical generation, which comprises mixing, in pharmacologically effective amounts, at least one lymphokine or cytotoxin from a mammalian species and at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor.
55. 2. The process according to claim 1 wherein the lymphokine or cytotoxin is interleukin-2, interferon- $\beta$ , tumor necrosis factor, or colony stimulating factor-1.

3. The process according to claim 1 or 2 wherein the lymphokine or cytotoxin is tumor necrosis factor-alpha.
4. The process according to any one of claims 1 - 3 wherein the biological modifier is selected from uric acid, bathionine sulphoximine, vitamin E, vitamin C, N-acetyl cysteine, a retinoid, glutathione, metronidazole, aspirin, indomethacin, ibuprofen, nordihydroguaiaretic acid, cis-8,11,14-eicosatrien-5-ynoic acid, synthetic prostaglandins, synthetic leukotrienes, and combinations of one or more of these modifiers.
5. The process according to any one of claims 1 - 2 or 4 wherein the lymphokine is interleukin-2.
6. The process according to claim 5 wherein the biological modifier is uric acid and the interleukin-2 is a desalanyl<sub>1</sub> mutein with a serine residue at position 125 of the native interleukin-2 molecule.
7. Use of pharmacologically effective amounts of at least one lymphokine or cytotoxin from a mammalian species and at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor for the production of a pharmaceutical composition for the therapeutic or prophylactic treatment of biological damage to a mammalian host caused by free radical generation.
8. The use of at least one biological modifier selected from a free radical scavenger or a metabolic inhibitor for the production of a pharmaceutical composition for enhancing the therapeutic index of at least one lymphokine or cytotoxin from a mammalian species in the therapeutic or prophylactic treatment of biological damage to a mammalian host caused by free radical generation.
9. Use according to claim 7 or 8 wherein the lymphokine or cytotoxin is selected from an interleukin, an interferon, a tumor necrosis factor, or a colony stimulating factor.
10. Use according to any one of claims 7 - 9 wherein the biological damage is cancer, infection, or damage caused by high oxygen tension, radiotherapy or chemotherapy.

30 Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Composition appropriée à l'administration à des hôtes mammifères pour le traitement thérapeutique ou prophylactique d'une lésion biologique de l'hôte provoquée par la formation de radicaux libres, laquelle composition comprend un mélange, en des quantités pharmacologiquement actives, d'au moins une lymphokine ou cytotoxine d'une espèce de mammifère et d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique.
2. Composition selon la revendication 1, dans laquelle la lymphokine ou cytotoxine est l'interleukine-2, l'interféron- $\beta$ , le facteur nécrosant des tumeurs ou le facteur stimulant la formation de colonies-1.
3. Composition selon la revendication 1 ou 2, dans laquelle la lymphokine ou la cytotoxine est le facteur nécrosant des tumeurs- $\alpha$ .
4. Composition selon l'une quelconque des revendications 1 à 3, dans laquelle le modificateur biologique est choisi parmi l'acide urique, la bathionine sulfoximine, la vitamine E, la vitamine C, la N-acétylcystéine, un rétinoïde, le glutathion, le méttronidazole, l'aspirine, l'indométhacine, l'ibuprofène, l'acide nordihydroguaiaretique, l'acide cis-8,11,14-eicosatriène-5-ynoïque, les prostaglandines synthétiques, les leukotriènes synthétiques et les combinaisons d'un ou plusieurs de ces modificateurs.
5. Composition selon l'une quelconque des revendications 1, 2 ou 4, dans laquelle la lymphokine est l'interleukine-2.
6. Composition selon la revendication 5, dans laquelle le modificateur biologique est l'acide urique et l'interleukine-2 est une désalanyl<sub>1</sub> mutéine avec un reste de sérine dans la position 125 de la molécule de l'interleukine-2 native.

7. Utilisation de quantités pharmacologiquement efficaces d'au moins une lymphokine ou d'une cytotoxine d'une espèce de mammifère et d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique, pour la production d'une composition pharmaceutique pour le traitement thérapeutique ou prophylactique d'une lésion biologique d'un hôte mammifère provoquée par la formation de radicaux libres.  
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8. Utilisation d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique, pour la production d'une composition pharmaceutique pour accroître l'index thérapeutique d'au moins une lymphokine ou une cytotoxine d'une espèce de mammifère dans le traitement thérapeutique ou prophylactique d'une lésion biologique d'un hôte mammifère provoquée par la formation de radicaux libres.  
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9. Utilisation selon la revendication 7 ou 8, dans laquelle la lymphokine ou la cytotoxine est choisie parmi une interleukine, un interféron, un facteur nécrosant des tumeurs ou un facteur stimulant la formation de colonies.  
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10. Utilisation selon l'une quelconque des revendications 7 à 9, dans laquelle la lésion biologique est un cancer, une infection, une lésion provoquée par une tension élevée d'oxygène, une radiothérapie ou une chimiothérapie.  
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**Revendications pour les Etats contractants suivants : ES, GR**

1. Procédé pour la préparation d'une composition appropriée à l'administration à des hôtes mammifères pour le traitement thérapeutique ou prophylactique d'une lésion biologique de l'hôte provoquée par la formation de radicaux libres, qui comprend le mélange, en des quantités pharmacologiquement actives, d'au moins une lymphokine ou cytotoxine d'une espèce de mammifère et d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique.  
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2. Procédé selon la revendication 1, dans lequel la lymphokine ou cytotoxine est l'interleukine-2, l'interféron- $\beta$ , le facteur nécrosant des tumeurs ou le facteur stimulant la formation de colonies-1.  
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3. Procédé selon la revendication 1 ou 2, dans lequel la lymphokine ou la cytotoxine est le facteur nécrosant des tumeurs- $\alpha$ .
35. 4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le modificateur biologique est choisi parmi l'acide urique, la buthionine sulfoximine, la vitamine E, la vitamine C, la N-acétylcystéine, un rétinoïde, le glutathion, le méthronidazole, l'aspirine, l'indométhacine, l'ibuprofène, l'acide nordihydroguaiarétique, l'acide cis-8,11,14-eicosatriène-5-ynoïque, les prostaglandines synthétiques, les leucotriènes synthétiques et les combinaisons d'un ou plusieurs de ces modificateurs.
40. 5. Procédé selon l'une quelconque des revendications 1, 2 ou 4, dans lequel la lymphokine est l'interleukine-2.
45. 6. Procédé selon la revendication 5, dans lequel le modificateur biologique est l'acide urique et l'interleukine-2 est une désalanyl mutéine avec un reste de sérine dans la position 125 de la molécule de l'interleukine-2 native.
50. 7. Utilisation de quantités pharmacologiquement efficaces d'au moins une lymphokine ou d'une cytotoxine d'une espèce de mammifère et d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique, pour la production d'une composition pharmaceutique pour le traitement thérapeutique ou prophylactique d'une lésion biologique d'un hôte mammifère provoquée par la formation de radicaux libres.
55. 8. Utilisation d'au moins un modificateur biologique choisi parmi un épurateur de radicaux libres ou un inhibiteur métabolique, pour la production d'une composition pharmaceutique pour accroître l'index thérapeutique d'au moins une lymphokine ou une cytotoxine d'une espèce de mammifère dans le traitement thérapeutique ou prophylactique d'une lésion biologique d'un hôte mammifère provoquée par la formation de radicaux libres.

9. Utilisation selon la revendication 7 ou 8, dans laquelle la lymphokine ou la cytotoxine est choisie parmi une interleukine, un interféron, un facteur nécrosant des tumeurs ou un facteur stimulant la formation de colonies.
- 5 10. Utilisation selon l'une quelconque des revendications 7 à 9, dans laquelle la lésion biologique est un cancer, une infection, une lésion provoquée par une tension élevée d'oxygène, une radiothérapie ou une chimiothérapie.

**Patentansprüche**

**10 Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE**

1. Mittel, das geeignet ist für die Verabreichung an Sägerwirte zur therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung des Wirtes, die durch Erzeugung freier Radikale verursacht wird, wobei das Mittel ein Gemisch in pharmakologisch wirksamen Mengen von mindestens einem Lymphokin oder Cytotoxin einer Sägerart und mindestens einem biologischen Modifikator umfaßt, der ausgewählt ist aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff.
- 15 2. Mittel nach Anspruch 1, wobei das Lymphokin oder Cytotoxin Interleukin-2, Interferon- $\beta$ , Tumornekrose-Faktor oder koloniestimulierender Faktor-1 ist.
- 20 3. Mittel nach Anspruch 1 oder 2, wobei das Lymphokin oder Cytotoxin Tumornekrose-Faktor- $\alpha$  ist.
4. Mittel nach einem der Ansprüche 1 bis 3, wobei der biologische Modifikator ausgewählt ist aus Harnsäure, Buthioninsulfoximin, Vitamin E, Vitamin C, N-Acetylcystein, einem Retinoid, Glutathion, Metronidazol, Aspirin, Indomethacin, Ibuprofen, Nordihydroguajaretsäure, cis-8,11,14-Eicosatrien-5-in-säure, synthetischen Prostaglandinen, synthetischen Leukotrienen, und Kombinationen von einem oder mehreren dieser Modifikatoren.
- 25 5. Mittel nach einem der Ansprüche 1 bis 2 oder 4, wobei das Lymphokin Interleukin-2 ist.
- 30 6. Mittel nach Anspruch 5, wobei der biologische Modifikator Harnsäure ist und das Interleukin-2 ein Desalanyl-1-mutein mit einem Serinrest in Stellung 125 des nativen Interleukin-2-Moleküls ist.
7. Verwendung von pharmakologisch wirksamen Mengen mindestens eines Lymphokins oder Cytotoxins einer Sägerart und mindestens eines biologischen Modifikators, ausgewählt aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff, für die Herstellung eines Arzneimittels zur therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung eines Sägerwirts, die durch die Erzeugung freier Radikale verursacht wird.
- 35 8. Verwendung von mindestens einem biologischen Modifikator, ausgewählt aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff, für die Herstellung eines Arzneimittels zur Verstärkung des therapeutischen Index von mindestens einem Lymphokin oder Cytotoxin einer Sägerart bei der therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung eines Sägerwirts, die durch Erzeugung freier Radikale verursacht wird.
- 40 9. Verwendung nach Anspruch 7 oder 8, wobei das Lymphokin oder Cytotoxin ausgewählt ist aus einem Interleukin, einem Interferon, einem Tumornekrose-Faktor oder einem koloniestimulierenden Faktor.
- 50 10. Verwendung nach einem der Ansprüche 7 bis 9, wobei die biologische Schädigung Krebs, eine Infektion oder eine Schädigung ist, die durch hohe Sauerstoffspannung, Radiotherapie oder Chemotherapie verursacht wird.

**Patentansprüche für folgende Vertragsstaaten : ES, GR**

- 55 1. Verfahren zur Herstellung eines Mittels, das geeignet ist für die Verabreichung an Sägerwirte zur therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung des Wirtes, die durch Erzeugung freier Radikale verursacht wird, umfassend das Mischen von pharmakologisch wirksamen Mengen von mindestens einem Lymphokin oder Cytotoxin einer Sägerart und mindestens

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einem biologischen Modifikator, der ausgewählt ist aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff.

2. Verfahren nach Anspruch 1, wobei das Lymphokin oder Cytotoxin Interleukin-2, Interferon- $\beta$ , Tumornekrose-Faktor oder koloniestimulierender Faktor-1 ist.  
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3. Verfahren nach Anspruch 1 oder 2, wobei das Lymphokin oder Cytotoxin Tumornekrose-Faktor- $\alpha$  ist.
4. Verfahren nach einem der Ansprüche 1 bis 3, wobei der biologische Modifikator ausgewählt ist aus Harnsäure, Buthioninsulfoximin, Vitamin E, Vitamin C, N-Acetylcystein, einem Retinoid, Glutathion, 10 Metronidazol, Aspirin, Indometacin, Ibuprofen, Nordihydroguajaretsäure, cis-8,11,14-Eicosatrien-5-in-säure, synthetischen Prostaglandinen, synthetischen Leukotrienen, und Kombinationen von einem oder mehreren dieser Modifikatoren.
5. Verfahren nach einem der Ansprüche 1 bis 2 oder 4, wobei das Lymphokin Interleukin-2 ist.  
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6. Verfahren nach Anspruch 5, wobei der biologische Modifikator Harnsäure ist und das Interleukin-2 ein Desalanyl-1-mutein mit einem Serinrest in Stellung 125 des nativen Interleukin-2-Moleküls ist.
7. Verwendung von pharmakologisch wirksamen Mengen mindestens eines Lymphokins oder Cytotoxins einer Säugerart und mindestens eines biologischen Modifikators, ausgewählt aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff, für die Herstellung eines Arzneimittels zur therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung eines Säugerwirts, die durch die Erzeugung freier Radikale verursacht wird.  
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8. Verwendung von mindestens einem biologischen Modifikator, ausgewählt aus einem Fänger freier Radikale oder einem Stoffwechsel-Hemmstoff, für die Herstellung eines Arzneimittels zur Verstärkung des therapeutischen Index von mindestens einem Lymphokin oder Cytotoxin einer Säugerart bei der therapeutischen oder prophylaktischen Behandlung einer biologischen Schädigung eines Säugerwirts, die durch Erzeugung freier Radikale verursacht wird.  
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9. Verwendung nach Anspruch 7 oder 8, wobei das Lymphokin oder Cytotoxin ausgewählt ist aus einem Interleukin, einem Interferon, einem Tumornekrose-Faktor oder einem koloniestimulierenden Faktor.  
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10. Verwendung nach einem der Ansprüche 7 bis 9, wobei die biologische Schädigung Krebs, eine Infektion oder eine Schädigung ist, die durch hohe Sauerstoffspannung, Radiotherapie oder Chemothe-riapie verursacht wird.  
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